

UNIVERSITE DE GENEVE
Département de pathologie

FACULTE DE MEDECINE
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Mouse bone marrow lymphocytes and their differentiation

THESE

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

par

Jean-Etienne RYSER

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Genève et Berne

Thèse N° 3396

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La Faculté de médecine, sur le préavis du professeur Pierre Vassalli, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

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Le doyen :
William Geisendorf

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Je remercie le professeur Pierre Vassalli sous la direction de qui ce travail a pu être accompli.

A LILIANE

INTRODUCTION

Nucleated cells of the bone marrow of adult laboratory rodents contain 20 % lymphocytes (BM lymphocytes), the majority of which are small and characterized by an unusually fast renewal time (1-5). In the present work, the small lymphocytes of the mouse bone marrow have been studied by immunofluorescence to determine their surface antigens (surface immunoglobulin and "differentiation" antigens), by autoradiography to determine their kinetics of labelling after exposure to ^3H -thymidine, and by a combination of these methods to follow their ability to differentiate after in vivo transfer to syngeneic recipients or in vitro, in cultures.

MATERIALS AND METHODS

Animals : 6 weeks to 8 months old mice of strains CBA/J, CBA/Ca, C3H, C57/B1, Balb/c, (CBA/J x C3H) F₁, (CBA/J x C57/B1) F₁, (C3H x C57/B1) F₁ and Swiss were obtained from Bomholtgard, Ry, Denmark and C. S. E. A. L. - C. N. R. S., Orléans-La-Source, France, or bred in our own colony.

Cell suspension : Mice were killed by exsanguination and BM cells were flushed from femoral and tibial cavities with cold Hank's balanced salt solution (BSS). A single cell suspension was then made by repeated aspiration in a glass pipette. After sedimentation of aggregates, cells were centrifuged in the cold and either used after two washings as a whole BM cell preparation or placed on sucrose gradients for further purification of lymphocytes. To purify spleen lymphocytes, spleen cell suspensions were placed on sucrose gradients in the same conditions. The red blood cells (RBC) of BM and spleen suspensions were lysed with ammonium chloride (0.75 % in 0.02 M Tris pH 7.4) : in the case of gradient selected cell populations, this step was performed after the sucrose gradient separation.

Sucrose gradient : A slight modification of the technique described by Osmond (6) was used. 200×10^6 cells were put in 1 ml BSS on a 10 ml 5 to 15 % sucrose gradient containing 20 % foetal calf serum (FCS, Difco Labs, Detroit, Michigan) and centrifuged 5 minutes at 270 g. A well defined band of slow sedimenting cells, RBC and lymphocytes, was aspirated from near the middle of the gradient and washed in a large excess of BSS. 3 to 5 % of the starting cell population were recovered, representing in the case of bone marrow 20 % of the lymphocytes loaded on the gradient.

Antisera : Preparation of the following antisera was as described previously (7) : AKR anti- θ C3H, rabbit anti-MSLA, -MBLA, -mouse Ig (RaMIg). -mouse K, μ , μ and α chains, and sheep anti-rabbit Ig (SaRIg).

The method of production and tests of specificity for immunofluorescence staining of rabbit anti-MSLA and anti-MBLA have been previously reported in detail (7) : in brief, anti-MSLA ("mouse specific lymphocyte antigen" : 8) is an antiserum raised against mouse thymocytes and absorbed with mouse liver homogenates and bone marrow cells until it fixes only on mouse thymocytes and T lymphocytes, and anti-MBLA ("mouse specific bone marrow derived lymphocyte antigen" : 9) is an antiserum raised against mouse spleen cells depleted in T lymphocytes and absorbed with mouse thymocytes and RBC until it recognizes only B cells among lymphocytes (7). Rabbit anti-human light chains was prepared by 6 weekly injections of 1-2 mg Bence Jones protein (mixture of LK and L λ chains) in complete Freund adjuvant for the three first injections, in phosphate buffered saline (PBS) for the three later. Most of the antisera were absorbed with mouse RBC and when necessary, with thymocytes.

Immunofluorescent staining and autoradiography : The techniques used for surface and intracellular staining and observations, alone or in combination with autoradiography, have been described previously (7). Cells were examined after cytocentrifugation (Shandon-Eliott cytocentrifuge) in 100 % FCS containing 0.03 M

NaN₃. For autoradiography, Ilford L 4 emulsion (Ilford, Basel, Switzerland) diluted 1/1 with distilled water, was used.

³H-thymidine labelling in vivo : (methyl-³H) thymidine (Amersham, 15-30 Ci/mmol) was diluted in BSS and 1 μ Ci per gram body weight injected twice, one hour apart, intraperitoneally.

Killing of cells by antisera and complement (C') : Guinea pig serum was used as source of C' after absorption with agar (10). Lymphocytes selected by sucrose gradients were incubated for 30 minutes at 37° at a concentration of 4 x 10⁶ cells/ml in BSS containing a 1/10 dilution of RaMIg and/or of each of the antisera used. After centrifugation, cells were resuspended for 30 minutes at 37° in a 1/6 dilution of guinea pig serum. Both incubations were performed in the presence of 0.03 M NaN₃, which was found to totally inhibit capping of surface antigens. Cells were then washed several times to diminish the proportion of dead cells. In each experiment, an aliquot of the cells to be used was stained with rhodamine-coupled SaRIg (Rho-SaRIg), and the absence of cells coated but not killed by antibodies verified in this way. Following treatment with RaMIg + C', the average cytotoxic index (judged by trypan blue exclusion and calculated as in ref. 7) was 31 % (+ 17) on purified BM lymphocytes (10 experiments) and 45 % (+ 9) on spleen cells (5 experiments). Usually, no residual living cell (trypan blue negative) still coated by RaMIg could be detected after incubation with Rho-SaRIg, except for a few experiments where some cells (less than 3 %) were found to be faintly labelled by Rho-SaRIg but not killed.

Cultures : 1 to 1.5 x 10⁶ cells were cultured in 1 ml medium RPMI 1640 containing 5 % FCS (Microbiological Associates, Bethesda, Md) at 37° in an humidified atmosphere of 5 % CO₂ in air. In some cultures, E. Coli lipopolysaccharide (LPS, Difco Labs, Detroit, Michigan, dialyzed against PBS and heated 10 minutes at 100° C) was added at a concentration of 20-40 μ g/ml, which was found to give a maximal stimulation of spleen cell cultures.

⁵¹Cr labelling of cells : 10⁸ nucleated cells were suspended for 45 minutes at 37° in 1 ml BSS containing 200 μ Ci ⁵¹Cr (Na chromate in isotonic solution, Würenlingen, Switzerland). The radioactivity of the injected washed cells and of the recipient organs were counted in a Packard scintillation spectrometer.

Assay for "colony forming cells" (CFU) : The technique described by Till and McCulloch (11) was used. 3 to 5 x 10⁴ cells were injected intravenously a few hours after 850 r irradiation of the recipients, which were sacrificed 8 to 9 days later.

Assay for "complement receptor lymphocytes" (CRL) : This assay was done according to Bianco et al (12). A 1/20 dilution of fresh normal mouse serum was used as source of C', and the rosette test done in RPMI medium containing 0.01 M EDTA. Normal spleen cells were included as control in every experiment.

RESULTS

BM small lymphocytes : study of surface antigens and receptors

The term "small lymphocyte" is applied in this study to small and medium sized lymphocytes, with a nuclear diameter up to $9\ \mu$ as measured after cyto-centrifugation and methanol fixation. It was observed that under these conditions, this size class corresponds to lymphocytes with a nuclear diameter of $8\ \mu$ or less on conventional smears. Lymphocytes with a nuclear diameter larger than $9\ \mu$ are classified as large lymphocytes or transitional cells.

BM small lymphocytes were studied by immunofluorescence either in whole BM cell preparations or after purification of the small lymphocyte populations by sedimentation through a sucrose gradient (6). This purification step results in a population containing 80 to 90 % of small lymphocytes, the contaminating cells consisting of metamyelocytes and mature granulocytes, erythroblasts (easily distinguishable from lymphocytes by Giemsa staining) and unclassified immature cells. Very few large lymphocytes or transitional cells were observed in this population.

The proportion of small lymphocytes bearing different surface antigen (Table I) was found very similar when judged on the whole BM populations and on the purified lymphocyte preparations. Thus, it was concluded that the purification step on sucrose gradient did not lead to a selection among the various BM small lymphocytes. This procedure was therefore used in most experiments, since in addition to its greater convenience for immunofluorescent and autoradiographic studies of small lymphocytes, it allows transfer experiments and cell cultures of populations only minimally contaminated by cells other than small lymphocytes. For comparison, spleen small lymphocytes were selected from spleen cell suspension on the same type of gradients, and populations containing 90-100 % small lymphocytes were obtained.

Immunofluorescent staining with anti-MSLA, anti-MBLA and anti-mouse Ig antisera, used alone or in combination, showed that about half of the BM lymphocytes do not bear any of these antigens (Table I). This is in marked contrast to what is observed with preparation of spleen cells (7,13) : gradient purified spleen small lymphocytes were found to be practically all MBLA⁺ or MSLA⁺. For convenience, the lymphocytes which are devoid of detectable surface differentiation antigens will be subsequently called "null" lymphocytes. These null lymphocytes bear apparently normal amounts of H₂ histocompatibility antigens (Table I). No significant difference in the proportion of BM null lymphocytes was observed in the various strains studied.

Table I

Immunofluorescent study of surface antigens of BM small lymphocytes

Antiserum used	% of lymphocytes stained *
anti-MBLA	34 (+ 12)
anti-MSLA **	8 (+ 5)
anti-MBLA + anti-MSLA	47 (+ 12)
anti-H ₂ ***	100
anti-mouse Ig	23 (+ 6)
anti-mouse K chains	23 (+ 6)
anti-mouse μ chains	11 (+ 3)
anti-mouse γ chains	0
anti-mouse α chains	1

* These are the average values and ranges (in parentheses) as observed in 20 experiments on inbred and outbred mice (6 weeks to 8 months of age) except for anti-K, - μ , - γ and - α chains antisera (3 experiments on Swiss mice) and for anti-H₂ histocompatibility antigens (1 experiment). All these experiments were done with gradient-purified BM lymphocytes. With whole bone marrow cells, the small lymphocytes showed the following stainings in 2 experiments : 3 % were stained with anti-MSLA, 25-27 % with anti-K serum and 46-63 % with anti-MBLA + anti-MSLA.

** Results obtained with AKR anti- θ C3H antiserum followed by Rho-RaMIg gave average values comparable to those obtained by adding the percentages of cells stained with anti-MSLA and with anti-mouse Ig.

*** BM lymphocytes from C57/B1 mice were incubated with a CBA anti-C57 alloantiserum, followed by a fluorescein-labelled rabbit anti-mouse Ig.

The lymphocytes bearing MBLA and surface immunoglobulin (sIg) were studied simultaneously by double immunofluorescent staining (7). All sIg⁺ cells were also MBLA⁺, but about 1/3 of the MBLA⁺ cells bore no detectable sIg : these MBLA⁺/sIg⁻ lymphocytes were less brightly stained with anti-MBLA antiserum than the sIg⁺ cells. Study of the sIg showed that the same percentage of cells were stained with anti-total mouse Ig and anti- μ chain antisera. Among heavy chains, practically only μ chains were found on the surface of BM lymphocytes, but the percentage of cells bearing μ chains was always found well below that of sk⁺ or sIg⁺ cells. Increasing the concentration of anti-heavy chains antisera did not reveal more cells bearing heavy chains. This discrepancy between the percentage of sIg⁺ or sk⁺ lymphocytes on one hand and of cells bearing detectable heavy chains on the other hand was never observed when purified spleen small lymphocytes were studied by immunofluorescence in similar conditions.

The presence on BM lymphocytes of a receptor for Fc was explored by the use of human heat-aggregated IgG according to Dickler and Kunkel (14). The cells fixing the aggregated human Ig were detected with a rhodamine-labelled rabbit anti-human light chain antiserum. The average result of three experiments was 33 % ($\pm$ 16) positive cells among purified BM lymphocytes. However, since the staining obtained was weak and the distinction between a very weakly stained cell and a negative one not always easy to make, this value might be somewhat inaccurate. In one experiment, the sIg⁺ cells were simultaneously detected by a fluorescein-labelled goat anti-mouse Ig antiserum. Aggregated human Ig was present on all the sIg⁺ cells (18 %). and, in addition, on 10-20 % of sIg⁻ cells.

Presence on the BM lymphocytes of the receptor for C' detected on peripheral B lymphocytes (12) was explored using sheep RBC coated with mouse C' (12). Whole spleen cell suspensions, or gradient purified spleen lymphocytes were used as controls. Two experiments gave identical results : about 30 % of the spleen lymphocytes formed rosettes, with 5-10 RBC per rosette; in contrast, there was little or no rosette formation (0-4 %) with BM small lymphocyte populations containing about 25 % of sIg⁺ cells, and the few rosettes observed were smaller (2-5 RBC per rosette). It can thus be concluded that, unlike the peripheral B lymphocytes, BM B lymphocytes have little or no receptor for C'.

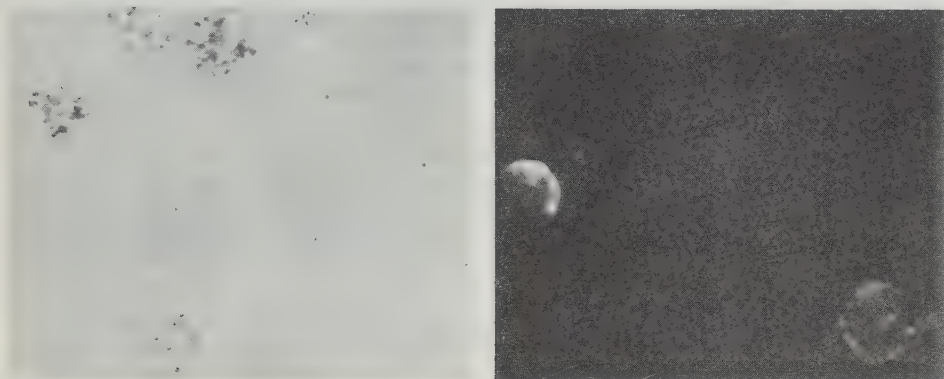
Autoradiographic and immunofluorescent studies of ³H-thymidine labelling kinetics of BM lymphocytes.

A number of kinetic studies using injections of ³H-thymidine have shown that the BM small lymphocytes of laboratory rodents are non-dividing, newly formed cells, arising from a population of rapidly dividing BM large lymphocytes called transitional cells (1-6). Mice were injected intraperitoneally with ³H-thymidine and killed one hour later. Whole bone marrow cell suspensions were incubated with a mixture of rabbit anti-MBLA and anti-MSLA antisera followed by Rho-SaRIg, cytocentrifuged and processed for autoradiography. Among the lymphocytes, the only ³H-thymidine labelled cells were large transitional cells, which were unstained by immunofluorescence. Two experiments were then performed, in which groups of mice were killed 40 hours after ³H-thymidine injection, and the BM small lymphocytes purified on sucrose gradients. Around 45 % of these lymphocytes were labelled by ³H-thymidine; more than 90 % of these

labelled lymphocytes were unstained by immunofluorescence (MSLA⁻/MBLA⁻, i.e. null), and about 80 % of the unstained lymphocytes were labelled by ³H-thymidine (Table II and fig. 1).

It can be concluded that most of the BM null lymphocytes are less than 40 hours old, while most of the BM B or T lymphocytes are more than 40 hours old.

Fig. 1



Gradient purified BM small lymphocytes, obtained from mice killed 40 hours after a pulse of ³H-thymidine, and incubated with a mixture of rabbit anti-MBLA and anti-MSLA antisera followed by Rho-SaRig. On the left is a radioautogram showing 4 ³H-thymidine labelled cells and 2 unlabelled cells. On the right, fluorescence of the same field : the 4 ³H-thymidine labelled cells are not fluorescent; the 2 fluorescent cells correspond to the 2 cells not labelled with ³H-thymidine.

Table II

Autoradiographic and immunofluorescent study of BM small lymphocytes obtained 40 hours after injection of ^3H -thymidine in vivo

	Exp. 1	Exp. 2
% of ^3H -thymidine labelled cells	42	46.5
% of MSLA ⁻ /MBLA ⁻ (null) cells	45	55
% of null cells among ^3H -thymidine labelled cells	91	92
% of ^3H -thymidine labelled cells among null cells	84	78

BM small lymphocytes from (CBA/J x C57)F₁ mice were purified on sucrose gradients, stained by immunofluorescence for MSLA and MBLA, and processed for autoradiography as described in the text. Autoradiographs were developed after 6-10 weeks.

Transfer of BM lymphocytes into syngeneic recipients

BM lymphocyte populations obtained from sucrose gradients were labelled in vitro with ^{51}Cr , with or without prior pretreatment by rabbit anti-mouse Ig, anti-MSLA and C' to enrich the population in null lymphocytes. The labelled lymphocytes were then injected intravenously into syngeneic recipients which were killed 20 or 48 hours later. The results of two representative experiments, showing the distribution of the radioactivity in different organs of the recipient mice, are found on Table III.

Table III

Distribution of ^{51}Cr labelled BM small lymphocytes after transfer

Exp.	Type of cells	Time of sacrifice	Spleen	Thymus	Lymph nodes*	Gut	Liver
1	untreated	20 h.	37	0	0.5	1.5	35
2	treated	48 h	28	0	0.1	0.5	34

1 to 2 x 10⁶ ^{51}Cr labelled BM small lymphocytes purified on a sucrose gradient (2 to 5 x 10⁴ cpm) were injected intravenously into syngeneic recipients. Results of 2 representative experiments out of 4, using CBA/J and Balb/c mice, are shown and expressed as % of the injected radioactivity recovered in the recipients' organs. The radioactivity recovered in the liver is interpreted as indicating the arrest of damaged cells in this organ.

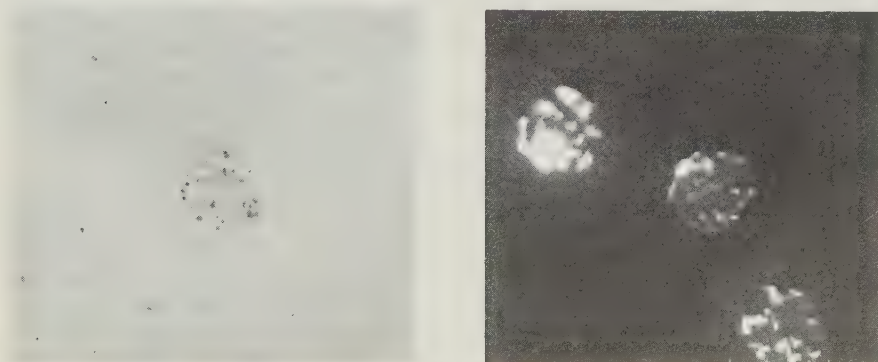
* Pooled peripheral and mesenteric lymph nodes

** Treated prior to transfer with anti-MSLA, RaMIg and C' as indicated in Methods.

With the exception of the spleen, there was little or no radioactivity in the organs rich in lymphoid cells (including the bone marrow, not shown on Table III). There was no indication of any migration of the injected cells into the thymus. In one experiment (not shown), the labelled BM lymphocytes were injected into recipients with a rapidly regenerating thymus (obtained by the injection 7 days before transfer of 9 mg of hydrocortisone to 6 weeks CBA/J mice); no radioactivity was recovered in the thymus 20 hours after transfer.

Since these experiments indicated that BM lymphocytes home mainly in the spleen after transfer, experiments were designed to analyze the surface antigens of labelled BM null lymphocytes recovered from the spleens of recipient mice 20 hours after transfer. The labelling of BM small lymphocytes was obtained either in vitro with ^{51}Cr , or in vivo with ^3H -thymidine ($2\mu\text{C/g}$ injected 40 hours before sacrifice). To eliminate all contaminating MSLA⁺ and MBLA⁺ cells (which are only a small minority among in vivo ^3H -thymidine labelled cells, as indicated above), the labelled BM lymphocytes were treated with rabbit anti-MBLA (or RaMIg), anti-MSLA and C', and the efficiency of the killing evaluated as described in Methods. Twenty hours after transfer of these labelled null lymphocytes, the recipient mice were killed, and aliquots of a cell suspension obtained from the spleen were incubated for surface immunofluorescence with anti-MSLA, anti-MBLA or anti-mouse Ig antisera. These various cell suspensions were cytocentrifuged and processed for autoradiography. The number of labelled cells detected on the autoradiographs was evidently very small, since the transferred labelled BM cells had been enormously diluted by the recipient's own spleen cells. Nevertheless, it was possible by immunofluorescence to analyze the surface antigens of about 80 ^3H -thymidine and 80 ^{51}Cr labelled cells (fig. 2 and Table IV).

Fig. 2



Immunofluorescent staining for surface Ig of a spleen cell suspension obtained from a recipient of ^3H -thymidine labelled BM null lymphocytes 20 hours after transfer. On the left is a radioautogram with a ^3H -thymidine labelled cell in the center. On the right, fluorescence of the same field showing that the ^3H -thymidine labelled cell bears sIg, although it is somewhat less brightly stained than the sIg⁺ lymphocytes of the host.

Table IV

Immunofluorescent staining of BM null lymphocytes recovered from recipient spleen 20 hours after transfer

Label of transferred null lymphocytes	Immunofluorescent staining of recipient spleen cells		
	MBLA	sIg	MSLA
^3H -thymidine	15/33	22/39	0/6
^{51}Cr	22/34	10/25	0/24

Results are expressed as the number of fluorescent and radiolabelled cells over the total number of radiolabelled cells scored in the cell preparations stained for the indicated antigen. (Thus, 15/33 in the MBLA column means that 15 radiolabelled cells were found to be fluorescent (MBLA⁺) out of a total of 33 radiolabelled cells scored on the preparations of cells stained for MBLA). Results of 3 transfer experiments were pooled in the case of ^3H -thymidine labelled cells, while results obtained with ^{51}Cr labelled cells represent a single transfer. Experiments were performed with Balb/c or C3H mice. 20 mice were used to obtain the BM null small lymphocytes used in a single transfer. Radioautographs were exposed for 6-10 weeks in the case of ^3H -thymidine and for 6 days in the case of ^{51}Cr labelled cells.

In the smears stained for MBLA or mouse sIg, about half of the labelled cells scored were also fluorescent, while in the smears stained for MSLA, none of the labelled cells was fluorescent. These experiments showed that BM null lymphocytes can, when transferred into a syngeneic recipient, differentiate within 20 hours into MBLA⁺ and sIg⁺ lymphocytes. On the other hand, there was no evidence that BM null lymphocytes can transform into T lymphocytes in the same conditions.

Autoradiographic studies were also performed on spleen sections of the recipients of ^3H -thymidine labelled null lymphocytes. Only a small number of labelled cells were observed per section (10-15 per transverse sections); they were located either on the edge of the peri-arteriolar sheathes or in the red pulp.

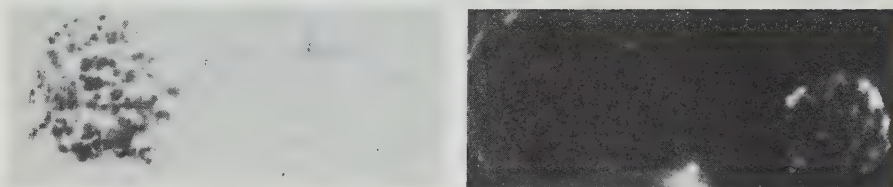
The possibility that BM null small lymphocytes can act as stem cells, or CFU, was explored by comparing the ability of purified BM lymphocytes and whole BM cells, or larger BM cells taken from the bottom of the sucrose gradient, to give rise to macroscopic colonies in the spleen after injection into irradiated syngeneic recipients (2 months old C3H or 8 months old CBA/Ca). Seven to nine recipients were used for each cell population. An average of 3.1 colonies (2 to 4) per 10^4 living injected cells were observed in recipients of whole BM cells or BM cells taken from the bottom of the gradient. In recipients of purified BM lymphocytes, the average value, in the same conditions, was 0.2 colonies (0 to 1).

Differentiation of BM lymphocytes in culture

Populations of BM small lymphocytes purified by sucrose gradients were cultured for 20, 40 and 60 hours in tubes containing $1-1.5 \times 10^6$ viable nucleated cells in 1 ml of medium RPMI 1640 with 5 % FCS. Aliquots of the cell population placed in culture were studied by immunofluorescence for surface Ig, and at the end of the culture, several tubes were pooled, the number of living cells determined, and immunofluorescent studies performed again. Results of representative experiments are given on Table V. The percentage of sIg⁺ lymphocytes often more than doubled after 20-40 hours of culture. However, since the recovery of living cells at the end of the culture varied between 20 to 40 %, this apparent increase in sIg⁺ lymphocytes might have resulted from the selective cell death of some sIg⁻ lymphocytes. Cultures were therefore established using lymphocyte populations depleted in sIg⁺ lymphocytes by prior incubation of the BM lymphocytes with RaMIg + C' (see Methods). The efficiency of the killing procedure was then ascertained by surface immunofluorescent staining of aliquots of the cell suspensions with Rho-SaRIg and Rho-RaMIg. Since these controls showed the absence of cells bearing rabbit antibody or mouse Ig, these cell populations can be called sIg⁻ lymphocytes. As soon as 20 hours after the beginning of the culture of BM sIg⁻ lymphocytes, about half of these cells had acquired sIg (Table V). A study of the various surface antigens present on the surviving cells from sIg⁻ BM lymphocytes cultured for 60 hours was performed with the following results : about 70 % were MBLA⁺, 50 % sIg⁺ and 40 % s μ ⁺ lymphocytes; s μ ⁺ and s α ⁺ cells were in negligible amounts, and about 5 % of MSLA⁺ lymphocytes were recovered. As controls, gradient purified spleen lymphocytes were also placed in the same culture conditions, with and without prior pretreatment to destroy sIg⁺ lymphocytes (Table V). In cultures containing sIg⁺ cells, the percentage of these lymphocytes did not increase, or decrease during the culture. More important, in cultures of spleen sIg⁻ lymphocytes, no sIg⁺ lymphocytes reappeared within 40 and 60 hours (85 % or more of the cells were MSLA⁺ at the end of the cultures). It was therefore concluded that sIg⁻ BM lymphocytes can differentiate rather rapidly in culture into sIg⁺ lymphocytes, and that, under these conditions, there are, as in the bone marrow itself, more MBLA⁺ than sIg⁺ cells, and more sIg⁺ cells than cells bearing detectable heavy chains.

The rapid appearance of sIg⁺ lymphocytes in cultures of BM sIg⁻ lymphocytes suggested that the acquisition of sIg may take place without cell division. To explore this point, a culture of sIg⁻ BM lymphocytes was performed for 60 hours in the continuous presence of ³H-thymidine (0.1 μ C/ml). This culture contained also LPS (see below). Immunofluorescent and radioautographic studies at the end of the culture showed that about 65 % of the cell were sIg⁺. 80 % of these sIg⁺ cells were small lymphocytes not labelled with ³H-thymidine (fig. 3), indicating that they had not divided during the culture; the other sIg⁺ cells were large cells, of which about 2/3 were radiolabelled.

Fig. 3



BM sIg^- lymphocytes, cultured for 60 hours in the presence of 3H -thymidine and LPS, then stained by immunofluorescence for surface Ig. On the left is a radioautogram showing a 3H -thymidine labelled blast next to an unlabelled small lymphocyte. On the right, fluorescence of the same field showing that the small lymphocyte bears readily detectable sIg, while the blast bears none or very little.

Since LPS is a B lymphocyte mitogen which also stimulates B lymphocytes to synthesize IgM molecules (15), the effect of adding LPS to culture of BM lymphocytes was explored in 3 experiments. About 40 % of the viable cells recovered after 40 and 60 hours were blasts and about 4/5 of them were sIg^+ . Among the untransformed small lymphocytes, the same percentage of sIg^+ cells (65 %) was found as in cultures without LPS. The cells obtained from these LPS stimulated cultures were cytocentrifuged, fixed, and stained for the detection of intracellular Ig chains by immunofluorescence; none of the blasts contained significant amounts of Ig chains. In contrast, in 2 experiments where gradient purified spleen small lymphocytes were cultured with LPS, 70-75 % of the recovered cells were blasts, almost all sIg^+ , while the remaining small lymphocytes were almost entirely sIg^- ; 15-20 % of the blasts contained, as detected by immunofluorescence, large amounts of intracellular Ig chains. These experiments indicated that BM lymphocytes are stimulated by LPS in culture, but that they do not react as the spleen B lymphocytes do, since the bone marrow cultures did show large numbers of untransformed sIg^+ small lymphocytes and since none of their blasts contained detectable intracellular Ig chains. Finally, in 4 experiments LPS was added to cultures of sIg^- BM lymphocytes; the percentage of blasts recovered at the end of the culture (up to 50 %) suggested that LPS did stimulate these cultures, but only about 20 % of these blasts were sIg^+ (fig. 3, Table V).

Table V

Immunofluorescent staining for surface Ig of cells recovered from lymphocyte cultures

Source of cells	Killing of sIg lymphocytes prior to cultures	Addition of LPS to cultures	% of sIg ⁺ cells before culture	after culture
BM lymphocytes	-	-	58	
	-	-	27	
	+	+	69	
	+	-	49*	
Spleen lymphocytes	+	+	0	44
	-	-	42	46**
	+	-	0	2
	+	-	0	2

All cell populations placed in culture were selected by sucrose gradients from the bone marrow or the spleen of Swiss mice. 3 experiments were done for each kind of cell. The cultures shown in this table were arrested after 40 hours, except when mentioned otherwise. Recovery of living cells at the end of the cultures varied between 20-40 % for BM lymphocytes and 25-45 % for spleen lymphocytes. The best recoveries were obtained in LPS containing cultures.

* Culture arrested after 20 hours

** About 30 % of sIg⁺ cells were found in 60 hours cultures.

DISCUSSION

Extensive kinetic studies by Everett, Osmond and others (1-6) have shown that the population of small lymphocytes present in the bone marrow of laboratory rodents has the following characteristics :1) it is made up of non-dividing cells, since these lymphocytes are not labelled shortly after a single injection of ^3H -thymidine, 2) most of these cells are newly formed and are rapidly replaced with a renewal time of about 3 days, as shown by the high percentage of labelled small lymphocytes observed after repeated injections or continuous perfusion of ^3H -thymidine, 3) this rapid renewal results from a high production of small lymphocytes locally in the bone marrow as seen after selective labelling of the BM cells by local injection of ^3H -thymidine in the tibial bone marrow cavity (2, 16). The precursors are a group of rapidly dividing large lymphoid, or "transitional" cells (1, 2, 17, 18, 19) which have been shown in culture to give rise to small lymphocytes (6), 4) most of the BM small lymphocytes derive from this local origin. There is practically no migration into the bone marrow of newly formed lymphocytes, since after ^3H -thymidine labelling of the short-lived cells of one animal in pairs of parabiotic rats, no labelled small lymphocytes appear in the bone marrow of the uninjected rat (20). Autoradiographic studies also showed that only 5-10 % of the BM small lymphocytes are long-lived (3, 5) and probably have entered the bone marrow from the pool of circulating long-lived lymphocytes.

The present study shows that a large fraction (about 50 %) of the mouse BM small lymphocytes bear neither the differentiation antigens specific for T or B lymphocytes (MSLA and MBLA), nor sIg. These cells, which are called for convenience null lymphocytes, are the first small lymphocytes arising in the bone marrow from the precursor transitional cells, as shown by the observation that forty hours after a pulse of ^3H -thymidine labelling these rapidly dividing precursors, almost all of the null lymphocytes are labelled while the lymphocytes bearing the B and T markers remain unlabelled. In vivo and in vitro experiments indicate that the null lymphocytes can rapidly acquire the membrane characteristics of B lymphocytes. In vivo, after transfer of labelled null lymphocytes into syngeneic mice, about half of the labelled lymphocytes recovered 20 hours later in the recipient spleens bear the MBL antigen and sIg. In vitro, cultures of BM small lymphocytes lacking sIg bearing cells (which had been killed by treatment with rabbit anti-mouse Ig serum and C') also show that a high proportion of lymphocytes acquire sIg within 20 hours. This process does not require cell division, as indicated by autoradiographic study of the sIg bearing lymphocytes appearing in cultures performed in the continuous presence of ^3H -thymidine. Control cultures of purified splenic small lymphocytes similarly depleted of sIg bearing cells did not show the appearance of sIg bearing cells.

Since, as already mentioned, the vast majority of the BM small lymphocytes are newly formed, rapidly renewed cells of local origin, it is reasonable to postulate that most if not all the BM B lymphocytes are cells which have just differentiated from null lymphocytes. Recent experiments of Osmond and Nossal (21) on the kinetics of labelling of mouse BM Ig-bearing lymphocytes strongly support this view. The first stage in the differentiation pathway towards the ma-

ture B lymphocyte is probably represented by the cells bearing only MBLA, but no sIg. Other evidence suggests that the differentiation of B lymphocytes is indeed a progressive process : the sIg present on BM lymphocytes seem to be quantitatively and qualitatively different from that found on B lymphocytes of other lymphoid tissues. Quantitatively, Osmond and Nossal (21), using a sensitive method of detection, observed that the sIg⁺ small lymphocytes vary in their capacity to bind antiimmunoglobulin much more widely in the bone marrow than in other lymphoid tissues. Qualitatively, the present experiments consistently detected an important discrepancy between the percentages of BM lymphocytes bearing K chains and of those bearing heavy chains (represented almost exclusively by μ chains). This discrepancy, which was not found in other lymphoid tissues, may indicate that the first immunoglobulin components appearing on the surface of differentiating B lymphocytes are isolated light chains, not yet assembled Ig molecules. Another possibility is that an important fraction of these BM sIg⁺ lymphocytes bear Ig molecules whose heavy chains are different from μ , γ and

chains. It must be emphasized that the differences between the B lymphocytes from the bone marrow and from peripheral lymphoid organs are not restricted to these variations in surface Ig. While all sIg⁺ lymphocytes in the bone marrow fix aggregated Ig, as it is the case for sIg⁺ lymphocytes from other sources (14) very few or no BM lymphocytes have receptors for C'. In contrast, the majority (but not all) of spleen B lymphocytes bear the C' receptors. LPS stimulates the appearance of IgM-bearing blasts in BM lymphocyte cultures, but by contrast with what is observed in spleen lymphocyte cultures, a significant number of the sIg⁺ small lymphocytes are not transformed into blasts, and blasts containing large amounts of Ig are not found. It thus seems clear that the B lymphocytes which differentiate in the bone marrow do not at once acquire all the properties of the B lymphocytes present in the peripheral lymphoid organs. There is indeed evidence, based on differences in migration pattern and tissue distribution, for the existence of two types of B lymphocytes, the "virgin" and "memory" cells (22). Since, according to these criteria, the bone marrow does not contain "memory" B lymphocytes (22), the properties exhibited by the spleen but not the BM B lymphocytes might be precisely those of the most differentiated lymphocytes, which have already been in contact with antigen. It is also possible that some of the BM sIg⁺ lymphocytes are not yet immunologically competent, notably those on which no heavy chains are detectable. Lafleur et al. have described in the mouse bone marrow "precursor" sIg⁺ B lymphocytes, which are not themselves functionally competent but can give rise to competent B lymphocytes after transfer into lethally irradiated syngeneic recipients. However, these "precursor" B lymphocytes were found to have a higher sedimentation velocity than the B lymphocytes, therefore to be presumably larger (23).

The conclusion that the B lymphocytes present in the bone marrow have differentiated locally from newly formed null lymphocytes leads to two important questions : is the differentiation of null lymphocytes into B lymphocytes taking place exclusively in the bone marrow? Do the null lymphocytes transorm only into B lymphocytes, or can they give rise to other cell lines as well?

Experiments involving the transfer of labelled BM null lymphocytes suggest that these cells can acquire, in vivo, sIg outside the bone marrow, and that the

spleen is the likeliest lymphoid organ where this differentiation can take place, since the null lymphocytes introduced in the circulation home preferentially in this organ. This does not indicate in itself, however, that this process of differentiation of null into B lymphocytes occurs on a significant scale in the spleen *in vivo*. Kinetic experiments on the renewal of BM small lymphocytes indicate that these cells do not all have the same transit time within the bone marrow (21) and suggest that some lymphocytes may leave the bone marrow before they had time to differentiate into B lymphocytes (16). In view of the relatively short time required for the transformation of null lymphocytes into B lymphocytes, even a rather large release of null lymphocytes from the bone marrow would not necessarily lead to a conspicuous percentage of null lymphocytes in the spleen. It is possible that part of the MBLA⁺/sIg⁻ (7) or of the θ^- /sIg⁻ (13) lymphocytes which are consistently found in the spleen represent B lymphocytes in the process of differentiating. It is however not very likely that most of these cells are the progeny of null lymphocytes recently emigrated from the bone marrow, since about 80 % of the θ^- /sIg⁻ spleen lymphocytes were found by Stobo et al. (13) to remain unlabelled after repeated injections of ³H-thymidine for 5 days. One may conclude that although it is difficult to establish to what extent lymphocyte differentiation from bone marrow precursors occurs outside the bone marrow, there is no evidence for a special lymphoid organ playing the role of a mammalian equivalent of the bursa of Fabricius, except for the bone marrow itself.

No evidence was found in the present experiments that the small null lymphocytes of the bone marrow can give rise to another cell line than the B lymphocytes. They do not have stem cell potentialities, as indicated by the CFU assay (11). They do not migrate to the normal or regenerating thymus, and no cell bearing the surface antigens characteristic of T cells were found among their progeny. On the other hand, it is possible that the small population of θ^- /sIg⁻ lymphocytes found in the spleen, and which are not newly formed (13) represent null lymphocytes which, after their migration from the bone marrow, have failed for an unknown reason to mature rapidly into sIg⁺ lymphocytes.

ABSTRACT

About 50 % of the mouse bone marrow (BM) small lymphocytes bear neither surface immunoglobulin (sIg) nor lymphocyte differentiation antigens (MBLA, MSLA or θ). These "null" lymphocytes are the most newly formed of the BM small lymphocytes. When purified populations of labelled BM null lymphocytes are transferred into syngeneic recipients, they migrate principally to the spleen and 20 hours after transfer about half of them have acquired characteristics of B lymphocytes (sIg, MBLA). After in vitro cultures, a high proportion of these cells acquire sIg and the MBL antigen, a process which does not require cell division. The null lymphocyte population does not contain detectable precursors of T lymphocytes or pluripotential stem cells. The BM B lymphocytes, which appear to derive directly from BM null lymphocytes, differ in several respects from the B lymphocytes found in peripheral lymphoid organs : a number of them bear light chains without detectable μ , γ or α heavy chains; they have little or no receptor for complement; their response to E. Coli lipopolysaccharide in culture is different from that of spleen B lymphocytes.

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